Localization and Purification of a Secretory Protein from the Esophageal Glands of *Meloidogyne incognita* with a Monoclonal Antibody

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ABSTRACT

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Strong labeling of secretory granules in dorsal esophageal glands of preparasitic second-stage juveniles and adult females of *Meloidogyne incognita* by affinity-purified monoclonal antibody $6D_4$ was observed by indirect immunofluorescence microscopy. This antibody also reacted weakly with secretory granules in the subventral esophageal glands of preparasitic juveniles. Postembedding immunogold labeling of ultrathin sections of juvenile esophageal regions revealed that the antigen was segregated around an electron-transparent core in the maxtix of secretory granules in the subventral glands. In the dorsal gland ampulla of adult females, specific gold labeling was localized principally in clumps in the cytoplasm adjacent to secretory granules. The antigen also was detected by immunofluorescence microscopy in stylet secretions of adult females. This secretory component was immunoaffinity purified and appeared to be a large-molecular-weight ($M_r > 212,000$) glycoprotein as indicated by its slow electrophoretic migration in 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and positive staining with periodic acid-Schiff reagent.

Plant-parasitic nematodes inject secretions synthesized in their esophageal glands into plant cells via a stylet when feeding. Little is known about the nature or function of these secretions (8). Esophageal gland secretions from second-stage juveniles (J2) of *Meloidogyne* species presumably transform recipient protophloem cells in roots of susceptible plants into a few specialized food cells, called giant cells, by modifying and regulating cell function and metabolism. These multinucleate giant cells subsequently are maintained by this sedentary endoparasite as the permanent nutrient source needed for their growth and reproduction. Secretions from these nematodes that induce and maintain this unique host response have not been purified; consequently, biologically active molecules have not been identified, nor is the role of the secretions in this very specialized host-parasite relationship understood.

Production of monoclonal antibodies specific for nematode secretions synthesized in the dorsal and/or subventral esophageal glands may enable secretory components involved in pathogenesis to be identified and characterized (9). The dorsal gland in sedentary endoparasitic nematodes is considered the most important esophageal gland in plant pathogenesis. Antibodies have been generated to secretory granules formed in the dorsal and subventral esophageal glands of *Meloidogyne incognita* (Kofoid & White) Chitwood (9) and *Heterodera glycines* Ichinohe (1). In both nematode species, antigens synthesized in the dorsal gland

of J2 also are produced in the dorsal gland of adult female nematodes. Antibodies raised with immunogens from *M. incognita* react with secretory granules in the esophageal glands of *M. javanica* (Treub) Chitwood and *M. arenaria* (Neal) Chitwood but not with antigens in *H. glycines* (9).

The objective of this study was to localize and purify a secretory component synthesized in the esophageal glands of *M. incognita* by means of a monoclonal antibody. A preliminary report has been published (10).

MATERIALS AND METHODS

M. incognita was propagated on greenhouse-grown tomato, *Lycopersicon esculentum* Mill. 'Rutgers.' Second-stage juveniles and adult females were collected as described previously (9).

Monoclonal antibody purification. The monoclonal antibody $6D_4$ was generated as described previously with crude homogenates of J2 as immunogens (9). Antibody isotype was determined by enzyme-linked immunosorbent assay (ELISA) with isotype-specific alkaline phosphatase-conjugated antibodies to mouse immunoglobulins (Southern Biotechnology Associates, Inc., Birmingham, AL). The antibody is an immunoglobulin M. The antibody was precipitated from spent hybridoma medium with 50% ammonium sulfate (14). The precipitate was redissolved in 100 mM Na-phosphate buffer, pH 7.8, containing 1 mM ethylenediaminetetraacetic acid and centrifuged at 10,000 *g* for 15 min to remove undissolved protein. An equal volume of 10 mM Na-phosphate buffer, pH 7.4, containing 500 mM NaCl and 0.02% NaN_3 (equilibration buffer) was added to the supernatant solution, and the mixture was applied on a 3 × 2.5 cm anti-mouse IgM-agarose affinity column (Sigma Chemical Co., St. Louis, MO) at a flow rate of 40 ml/hr at 5 C. The effluent was recycled to the column at least three times. Next the column was washed with the equilibration buffer until the A_{280} was less than 0.002. The bound antibodies then were eluted with a buffer consisting of 100 mM glycine and 150 mM NaCl, pH 2.4. One-milliliter fractions were collected in test tubes containing 0.2 ml of neutralizing buffer (500 mM Tris, pH 8.5), and those containing the antibodies were pooled and concentrated by ammonium sulfate precipitation as described.

Immunofluorescence. Preparasitic J2 and adult female nematodes (25–33 day old) were fixed and processed for indirect immunofluorescence staining with monoclonal antibody $6D_4$ following the procedure described previously (9). Nematode sections were viewed with differential interference contrast optics and a ×40 oil immersion objective of an Olympus BH-2 fluorescence microscope.

Stylet secretions. Viable adult female nematodes were dissected from galled 35-day-old tomato roots and incubated in 100 mM NaCl containing 1 mg of streptomycin sulfate/ml (Sigma Chemical Co.) for 1 hr. Seven nematodes were carefully aseptically mounted in a solidified drop of 0.7% (w/v) Noble water agar on a 24 × 40 mm glass coverslip. Each nematode was positioned in the agar so that its head was out of the agar and oriented toward the edge of the agar drop. Immobilized female nematodes on the coverslip were placed in a perfusion chamber (15), which was filled with 500 µl of the NaCl-streptomycin sulfate solution. The perfusion chamber was incubated in a moist container at room temperature for 3 days to allow stylet secretions to accumulate at the oral aperture of the nematodes. A modified fluorescein isothiocyanate (FITC)-immunofluorescence procedure (9) was used to treat intact, nonfixed females and their adhering stylet secretions within perfusion chambers. Nonspecific binding sites in the stylet secretions were blocked by adding 500 µl of 10% goat serum (Sigma Chemical Co.) in phosphate-buffered saline (PBS), pH 7.4 (137 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 8.1 mM Na_2HPO_4) to the chamber for 1 hr. Secretions next were incubated in monoclonal antibody $6D_4$ (10 µg/ml) diluted with 1% bovine serum albumin (BSA) in PBS for 2 hr followed by three 15-min washes in PBS. FITC-IgG goat anti-mouse conjugate (Sigma Chemical Co.) diluted 1:500 with Tris-saline containing 0.2% Triton-X 100 and 3% BSA was added to the perfusion chamber and incubated for 2 hr in the dark at room temperature. Specimens were washed three times in PBS followed by a final distilled water wash and viewed with an Olympus fluorescence microscope. As a control, the monoclonal antibody $6D_4$ was replaced with a monoclonal antibody ($3H_{11}$) specific for secretory granules in subventral glands of J2 (9).

Immunogold electron microscopy. Freshly hatched J2 and 26- to 32-day-old adult females were fixed at room temperature for 1 hr in freshly prepared 50 mM phosphate buffer, pH 6.8, containing 2% paraformaldehyde and 0.2% glutaraldehyde. Nematodes were punctured with a needle and cut in half after fixation for 15–30 min. Anterior sections of nematodes were embedded in a 0.25-mm-thick pad of 2% Noble agar. Small pieces of agar containing individual nematode sections were transferred to a vial with 50 mM phosphate buffer, pH 6.8. The buffer was replaced with 50 mM lysine in phosphate buffer and incubated overnight at 4 C.

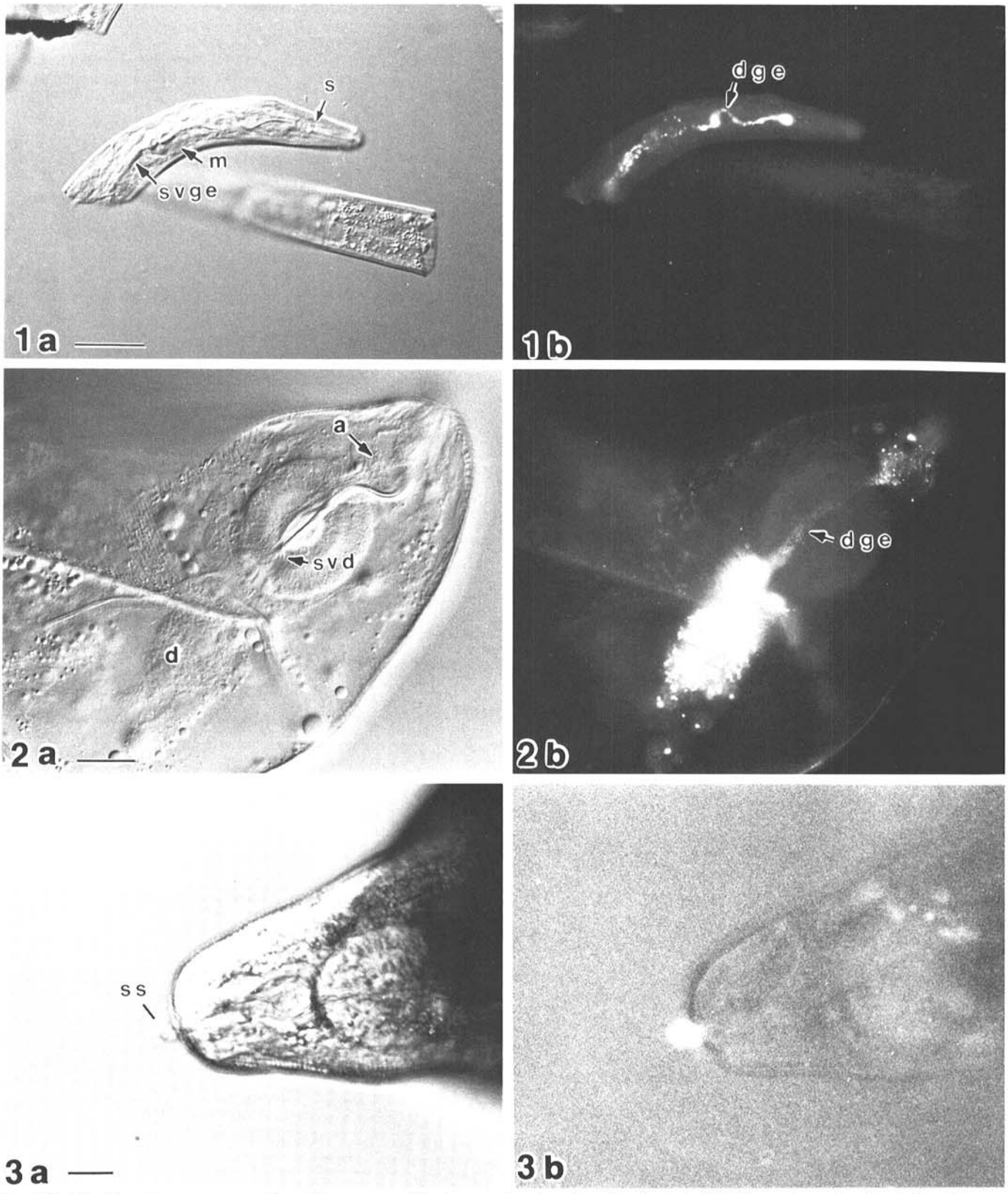
Nematode sections were dehydrated through a graded 30–90% series of ethyl alcohol and then infiltrated and flat embedded in LR White medium-grade resin (Polysciences, Inc., Warrington, PA). Flat embedding was accomplished by transferring 8–10 agar pieces with nematode sections to the center of a 0.5-ml droplet of resin on a microscope slide. Two #1 coverslips for J2 sections and three for adult females were placed at each end of the slide to support a microscope slide which was carefully lowered onto the coverslips. The microscope slides had been triple coated with a tetrafluoroethylene release agent (Miller Stephenson Chemical, Danbury, CT) (16). The microscope slides containing the specimens were placed in a modular incubator chamber (Markson, Phoenix, AZ) which was purged with nitrogen; the resin was heat polymerized by placing the chamber in an oven at 50 C for 24 hr. The two microscope slides were separated after the sample block had been placed at −20 C for 2 min.

The flat-embedded samples were examined by light microscopy, excised with a scalpel, and oriented and glued onto blank resin blocks (12). Ultrathin (75–80 nm) sections were cut with a diamond knife on a Reichert-Jung Ultracut E microtome (C. Reichert Optische Werke AG, Wien, Austria), collected on Formvar-coated gold slot grids, and processed for on-grid immunolabeling (17) by floating grids, sections down, on 30-µl drops in polystyrene petri dishes at room temperature as follows: 10 min on 10% H_2O_2, 5 min on PBS, 2 hr on 3% BSA in PBS, overnight in a moisture chamber on monoclonal antibody $6D_4$ diluted to 1 µg/ml of 0.1% BSA-PBS, three 10-min washes with 1% BSA-PBS, 2 hr on goat anti-mouse IgM or IgG (H & L) coupled with 10-nm colloidal gold (Jansen Life Sciences Products, Piscataway, NJ) diluted 1:20 with 0.1% BSA-PBS, three 5-min PBS washes, and a final wash with deionized water. For control sections, monoclonal antibody $6D_4$ was omitted or replaced with an irrelevant antibody. The immunolabeled sections were stained with aqueous uranyl acetate and lead citrate and observed and photographed in a Zeiss EM-10A transmission electron microscope operated at 60 kV.

Preparation of affinity column. Affi-gel 10 and Affi-gel hydrazide (Hz) (Bio-Rad Laboratories, Richmond, CA) were coupled with at least 3 mg of monoclonal antibody $6D_4$/ml of support. Affi-gel Hz was coupled according to the protocol of Bio-Rad Laboratories. Coupling of Affi-gel 10 was made as follows. Approximately 3 ml of the gel was placed in a small prechilled fritted glass funnel and quickly washed three times with ice-cold distilled water. The moist gel cake was transferred in a premarked 4-ml glass vial which then was spun briefly in a clinical centrifuge to pack the gel. Excess water was drained, and an equal volume of monoclonal antibody $6D_4$, previously equilibrated in 200 mM $NaHCO_3$, 300 mM NaCl, pH 8.0 (coupling buffer), was added to the gel. The mixture was agitated gently on a rocker overnight at 4 C. The coupled gel then was transferred into a 0.7 × 10 cm glass column and washed with five bed volumes of 100 mM $NaHCO_3$, 150 mM NaCl, pH 8.0, and the equilibration buffer (10 mM Na-phosphate, 140 mM NaCl, pH 7.4) containing 0.02% NaN_3 until A_{280} was less than 0.002. The affinity columns were maintained wet at 5 C.

Antigen purification. Two to three milliliters of packed J2

nematodes was frozen at −80 C and powdered in a mortar and pestle with the aid of liquid nitrogen. An equal volume of 100 mM Na-phosphate, pH 7.4, buffer containing 154 mM NaCl and 0.1% Triton-X 100 was added to the powdered nematodes and triturated in a glass homogenizer. The homogenate was centrifuged twice at 10,000 *g* for 15 min followed by filtration of the supernatant through a Millex HA 0.45-μm filter unit (Millipore, Bradford, MA) to remove insoluble materials. Protein con-

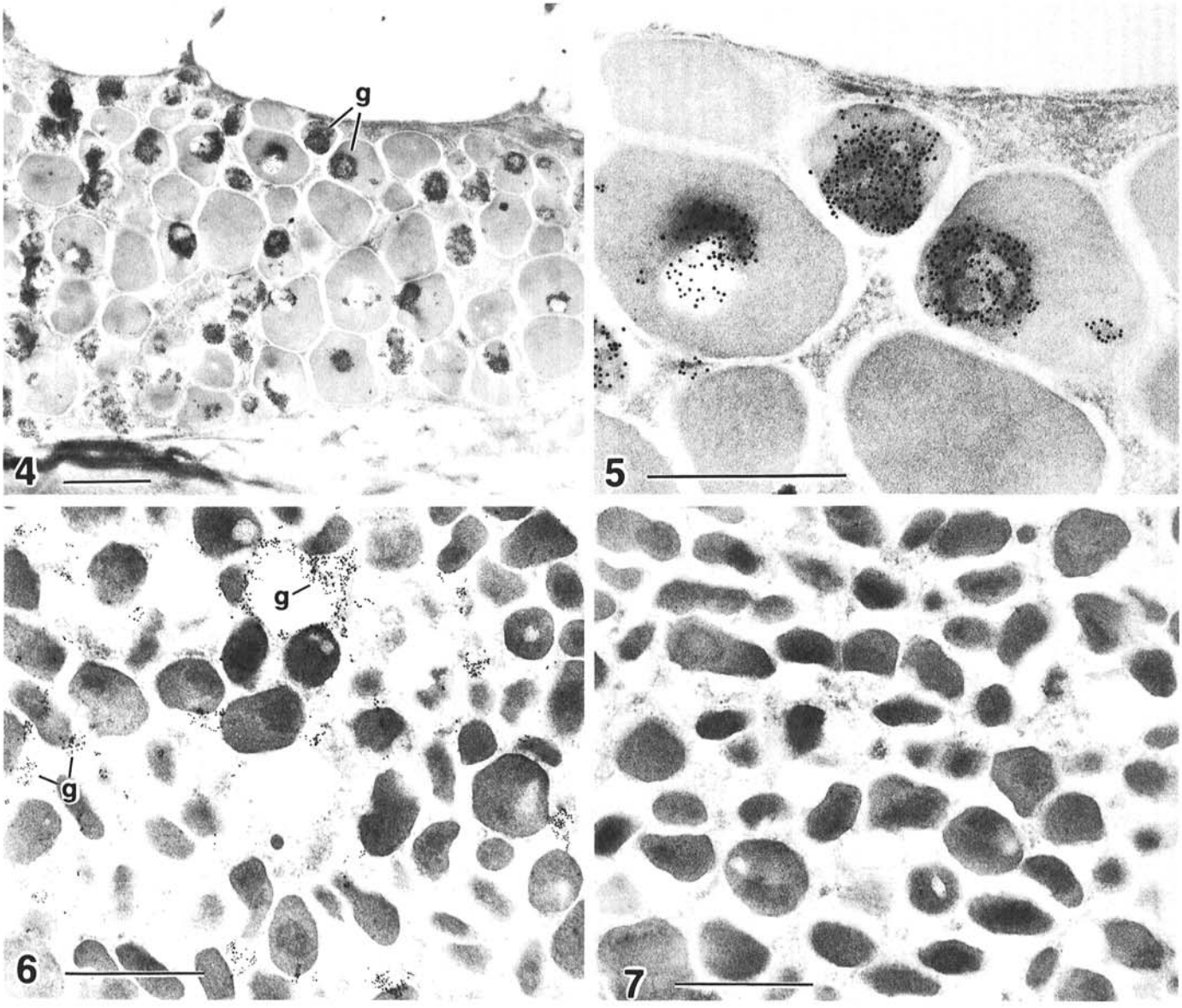


Figs. 1–3. Paired interference contrast **(A)** and fluorescence **(B)** micrographs of esophageal regions of *Meloidogyne incognita* after indirect immunofluorescence staining with the $6D_4$ monoclonal antibody. **1,** Immunofluorescent staining of secretory granules in the cytoplasmic extensions of the dorsal and to a lesser degree the subventral glands of a preparasitic second-stage juvenile section. The subventral gland extensions (svge) terminate in the metacorpus (m), and the dorsal gland extension (dge) terminates near the base of the stylet (s). **2,** Immunofluorescent staining of granules in the dorsal gland (d) and its extension (dge) and ampulla (a) in an adult female nematode. Note the absence of staining associated with the subventral gland duct (svd). **3,** Immunofluorescent staining of stylet secretions (ss) of an adult female nematode. Scale bars = 20 μm.

centration was determined by the method of Bradford (5). The clarified homogenate was applied to either monoclonal antibody $6D_4$-coupled Affi-gel 10 or Affi-gel Hz columns following the chromatographic steps described in monoclonal antibody purification. Fractions containing the monoclonal antibody $6D_4$-specific antigen were pooled and concentrated, with an Amicon Centricon 10 concentrator (Amicon, Danvers, MA).

Electrophoresis and immunoblotting. Purified antigen was analyzed in 0.75-mm-thick, 7.5% discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system (11) with a mini Protean II cell (Bio-Rad Laboratories). About 10 μl of the protein preparation, containing about 80 μg of protein, was mixed with three volumes of the sample buffer, heated to 100 C for 4 min, loaded into the corresponding wells, and electrophoresed at 200 V for 45 min. Molecular weight marker proteins were those from a high-M_r calibration kit (Pharmacia, Piscataway, NJ). After electrophoresis, proteins were detected by staining with silver nitrate. Periodic acid-Schiff's reagent staining for carbohydrate also was performed according to Pharmacia protocol. For immunoblotting, gels were incubated in 48 mM Tris and 39 mM glycine, pH 9.2, containing 20% methanol and 3.75 ml of 10% SDS/L (4) or 25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol (18) transfer buffer for 20–30 min. Protein transfer to nitrocellulose membrane was performed in a semi-dry electrophoretic transfer cell (Bio-Rad Laboratories) at 15 V for 30 min.

After transfer, the membrane was soaked in TBS (200 mM Tris, 500 mM NaCl, pH 7.5) for 15 min to remove SDS, and nonspecific binding sites were blocked with 3% gelatin in TBS for 1–1.5 hr. The membrane then was given three 5-min washes in TTBS (TBS + 0.05% Tween 20) before incubating overnight in a solution containing 1 μg of monoclonal antibody $6D_4$/ml of antibody diluent (1% gelatin in TTBS) with agitation. The membrane was washed in TTBS, followed by 1 hr incubation in 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgM in antibody diluent. The excess conjugate was removed by two 5-min washes in TTBS, one 5-min wash in TBS, and one 5-min wash in bicarbonate buffer (100 mM $NaHCO_3$, 1 mM MgCl), pH 9.8. Protein bands were visualized by incubating membranes in a p-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Bio-Rad Laboratories) solution in bicarbonate buffer for 15–30 min.



Figs. 4–7. Post-embedding immunogold labeling of a secretory component synthesized in the esophageal glands in *Meloidogyne incognita*. **4, 5,** Labeling of secretory component sequestered in secretory granules in a subventral gland of a preparasitic second-stage juvenile. Gold particles (g) are concentrated in a zone around an electron-transparent core present in the matrix of secretory granules. **6,** Labeling of the secretory component in the dorsal gland ampulla of an adult female nematode. Gold particles (g) are concentrated principally in clumps in the cytoplasm adjacent to secretory granules. **7,** Control section of dorsal gland ampulla of an adult female nematode treated as in Figure 6, but the primary antibody was replaced with an irrelevant monoclonal antibody. No specific staining occurred. Scale bars = 1.0 μm in Figure 4 and 0.05 μm in Figures 5–7.

RESULTS

Immunofluorescence. An antibody, $6D_4$, was generated that has specificity for secretory granules formed in the esophageal glands of J2 and adult female nematodes. In J2 nematodes, affinity-purified monoclonal antibody $6D_4$ reacted strongly with secretory granules in the dorsal gland and its cytoplasmic extension, which terminates near the base of the stylet (Fig. 1A and B). The antibody weakly bound to granules present in the subventral glands and their cytoplasmic extensions, which terminate immediately behind the pump chamber in the metacorpous. In adult females, monoclonal antibody $6D_4$ reacted with secretory granules throughout the dorsal gland lobe and its cytoplasmic extension, as revealed by the immunofluorescent staining pattern (Fig. 2A and B). No secretory granules were observed at the subventral gland ducts (Fig. 2A), nor was any immunofluorescent staining associated with these structures in adult females (Fig. 2B).

Stylet secretions accumulated at the oral aperture of living adult female nematodes incubated in a perfusion chamber (Fig. 3A). Secretions treated with monoclonal antibody $6D_4$ and the FITC-immunofluorescence procedure stained with FITC, indicating that the antigen was secreted through the stylet of the nematode (Fig. 3B). No staining of stylet secretions occurred when monoclonal antibody $6D_4$ was replaced with a monoclonal antibody ($3H_{11}$) specific for secretory granules formed in the subventral glands of J2 nematodes.

Immunogold labeling. Specific gold labeling with monoclonal antibody $6D_4$ was localized in the matrix around an electron-transparent core in secretory granules formed in the subventral glands of preparasitic J2 nematodes (Fig. 4). Dense labeling with gold particles commonly occurred in a spiral pattern in this localized area of the granule matrix (Fig. 5). In the ampulla of the dorsal gland of adult female nematodes, gold particles were localized principally in clumps in the cytoplasm adjacent to secretory granules (Fig. 6). There was no preferential localization of gold particles to the electron-dense matrix of dorsal gland secretory granules. In control sections, no gold labeling occurred when monoclonal antibody $6D_4$ was omitted or when it was replaced with an irrelevant antibody of the same immunoglobulin class (Fig. 7).

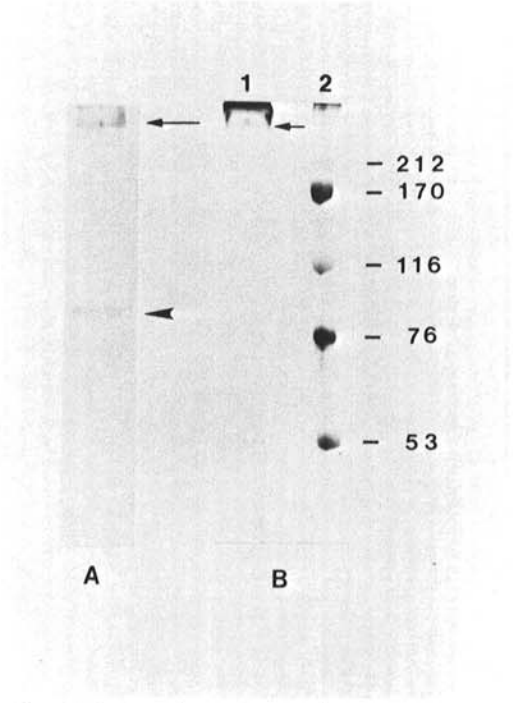


Fig. 8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of an immunoaffinity-purified secretory protein from *Meloidogyne incognita*. **A,** Immunoblot probed with monoclonal antibody $6D_4$: secretory protein (arrow) and heavy chain of monoclonal antibody (arrowhead). **B,** Silver-stained 7.5% polyacrylamide gel. Lane 1, secretory protein (arrow); lane 2, molecular-weight markers in order of decreasing mass: myosin, α_2-macroglobulin, β-galactosidase, transferrin, and glutamic dehydrogenase. Numbers to the right are molecular mass ($\times 10^3$ Da) of marker proteins in lane 2.

Antigen purification. The secretory protein from homogenates of J2 was selectively eluted from immunoaffinity columns of either Affi-gel 10 or Affi-gel Hz. The protein, as visualized by silver-stained SDS-PAGE gels, was resolved as one band at M_r much greater than 212,000 (Fig. 8). The protein also reacted with periodic acid-Schiff reagent, indicating that it was a glycoprotein (data not shown). When electrotransferred on nitrocellulose membrane, the secretory protein was detected by monoclonal antibody $6D_4$ (Fig. 8).

Recovery of the secretory protein was quite low, due in part to the small proportion of the protein in J2 homogenates. In addition, Affi-gel 10 and Affi-gel Hz may not be the appropriate support for large-molecular-weight ligands like IgM. Monoclonal antibody $6D_4$ coupled to Affi-gel 10 bled with the eluted secretory protein (Fig. 8). With Affi-gel Hz, no bleeding of the antibody was observed; however, recovery of the secretory protein was lower than with Affi-gel 10.

DISCUSSION

Until now, no secretion whose origin has been established in the esophageal glands has been purified from any plant-parasitic nematode. The secretrory protein purified in this study was synthesized in the dorsal and subventral esophageal glands of J2 and only the dorsal gland of adult females, indicating that it was synthesized only in the dorsal gland throughout the life cycle of the nematode. The subventral glands in J2 stage nematodes shrink with the onset of parasitism (3), and the dorsal gland becomes the predominant gland in adult females. Furthermore, the secretory activity of subventral glands decreases as J2 mature. This conclusion is supported by monoclonal antibody $6D_4$ and other antibodies that have specificity for antigens in subventral glands of preparasitic J2 (9) but do not detect their complementary antigens in the esophagus of adult females. Although there is convincing evidence supporting a role for the dorsal gland in parasitism (8), the physiological function of the subventral glands and what role, if any, these glands have in parasitism remain obscure.

The secretory protein was packaged in membrane-bound secretory granules formed in the subventral esophageal glands. These granules were formed by budding from Golgi bodies. Because this secretory protein was segregated in a specific domain of subventral gland granules, undoubtedly other secretory components were present in the matrix. In most secretory cells (13), secretory granules contain at least two types of molecules: passenger proteins destined for export out of the cell, and specific proteins involved in granule transport and exocytotic secretion. Localization of the secretory protein in the dorsal gland ampulla of adult females did not demonstrate clearly that the protein was packaged in secretory granules, although the protein always was localized adjacent to granules. This pattern of distribution possibly indicates that the protein had been released from the granules.

Presence of the secretory protein in secretions accumulating at the oral aperture of living adult female nematodes provided evidence that it was secreted through the stylet and therefore is potentially important in plant pathogenesis. Stylet secretions collected from adult females of *M. arenaria* and *M. incognita* contained nine proteins, although the specific origin, whether from the esophageal glands or amphids, of the proteins was not established (19). Our data showed that one component of stylet secretions from adult females of *M. incognita* was synthesized in the dorsal esophageal gland.

Purification of the secretory protein revealed that it was a high-molecular-weight glycoprotein. Stylet secretions of adult females previously have been shown to contain glycoproteins (2,19), and, when separated by SDS-PAGE, one component had a high

molecular weight (19). The majority of secretory proteins undergo post-translational chemical modifications, usually in the form of glycosylation, during their passage through Golgi bodies in secretory cells (6).

Monoclonal antibodies specific for stylet secretions of plant-parasitic nematodes may enable those secretions that have a role in plant pathogenesis to be identified and characterized. Research is in progress to determine the function of the secretory protein purified in this study and to identify its subcellular location in nematode-induced giant cells. These antibodies also could lead to development of novel root-knot-resistant plants by transfer of appropriate immunoglobulin coding sequences to a higher plant (7). The plant itself then would synthesize antibodies to a nematode secretory component. Precipitation of nematode secretory components by the antibodies might inhibit nematode development on the transgenic plant.

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